Investigation of the role of DNA Methyltransferases in Autophagy Gene Expression and Function in Idiopathic Pulmonary Fibrosis (IPF)

Research Thesis

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Abstract:

Idiopathic pulmonary fibrosis (IPF) is characterized by a significant increase in collagen deposits in lung extracellular matrix (ECM) compared to non-IPF patients. Excessive collagen deposition is essential to the pathology of IPF and affects the fibrotic response to injury in lung tissue. Collagen turnover is regulated by autophagy, an intracellular mechanism that involves lysosomal degradation of unnecessary or dysfunctional cell components. It has been reported that autophagy is unsuccessful despite pathway activation in lungs of IPF patients. Previous work demonstrated that combination treatment of IPF cell lines with 5'-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, and rapamycin, an mTOR inhibitor, restored expression of key autophagy-regulating genes through decreased promoter CpG island methylation. Using primary lung fibroblasts from IPF patients and IPF fibroblast cell lines, we performed knockdown studies of each DNMT-1, 3A, and 3B mRNAs. Initial results show a marked reduction of DNMT-1 mRNA (84% reduction in both normal and severe IPF fibroblasts), DNMT-3A mRNA (68% reduction in normal fibroblasts relative to 83% in severe fibroblasts), and DNMT-3B mRNA (42% reduction in normal fibroblasts compared to 49% in severe IPF fibroblasts). Interestingly, as a result, when comparing expression of the 3 key autophagy-regulating genes BECN1, ATG5, and ATG12, it was targeting of DNMT-3B, and not DNMT-1 as hypothesized, that increased expression of these autophagy genes. This data suggests that DNMT-3B plays a regulatory role in restoring autophagy to IPF fibroblasts. In order to verify these data, we are currently investigating autophagy function in normal and IPF fibroblasts after siRNA targeting of DNMTs. Understanding the relationship between the functional responsibilities between DNA methylation moieties like DNMTs and histone deacetylases (HDACs), and their regulation of autophagy genes and function could identify new therapeutic targets for patients with IPF.

Introduction:

The cause of Idiopathic Pulmonary Fibrosis (IPF) is, by definition, unknown. However, recent research has led a belief that IPF may be caused more by mechanisms representing aberrant wound healing more than chronic inflammation, which was previously thought to underlie the pathogenesis of IPF. Most of the recent drug activities involve inhibiting uric acid levels and attempting to reduce excess collagen deposits in the lung extracellular matrix (ECM). The prevalence of the disease is high for a lung disease, as IPF represents the most common cause of death from any progressive lung disease.

My project focuses on mechanisms responsible for dysfunctional collagen turnover in IPF lungs and its importance in the pathology of the disease. As discussed above, one mechanism for successful collagen turnover is autophagy. It has been reported that, in lungs of IPF patients, functional autophagy is inhibited despite pathway activation. Previous work by the Marsh laboratory showed that multiple autophagy genes (ATG5, ATG12, LC3-I, LC3-II) were hypermethylated.
(excessive promoter CpG island methylation) in IPF lung samples compared to samples from non-IPF lungs. At the same time, it has been shown that DNA methyltransferases (DNMTs) are significantly overexpressed in IPF lungs, with an unclear relationship to autophagy. This led us to hypothesize that excessive DNMT activity was repressing autophagy-related gene expression, activation of autophagy function, and ultimately collagen turnover. We hypothesized that knockdown of specific DNMTs by siRNA targeting may restore autophagy-related gene expression and function in IPF fibroblast cell lines reflecting autophagy function essential for healthy lung function.

Methodology:

Gene expression:
To assay mRNA expression, total RNA was collected from normal and IPF fibroblast cell lines by TRIzol, synthesized into cDNA using SuperScript III Reverse Transcriptase from Life Technologies and subsequently quantified by qRT-PCR. mRNA expression for all genes between samples were compared to housekeeping genes CAP1 and RPL4.

DNA methylation:
To assay for DNA promoter CpG island methylation, genomic DNA was isolated and quantified using the EpiTect II DNA methylation kit.

Autophagosome formation:
To show autophagosome formation, cells were immunostained with DAPI (for cell nuclei) and GFP (specifically targeting autophagic vacuoles) and imaged through a live imaging microscope.

Protein expression:
For protein expression of autophagy genes, samples were subjected to gel electrophoresis transfer and immunoblotted with specific antibodies.

Primary fibroblasts and fibroblast cell lines:
Cell lines were acquired from the ATTC (American Type Culture Collection) and patients across the United States.

Results:

Lung tissue from IPF patients have significantly reduced levels of key autophagy genes such as LC3-I, LC3-II, ATG5, and BECN1 as compared to normal controls.
In addition, autophagy protein levels are significantly reduced in IPF lung tissue compared to normal controls, confirming what is observed at the mRNA level.

**Figure 1.** Decreased autophagy gene expression in human IPF lung tissue compared to normal controls. Control (n=11), >80% FVC (n=8), 50-80% FVC (n=8) and <50% FVC (n=9), *p<0.005 compared to control tissue. This figure shows that IPF lung tissue, especially in its most severe form, has significantly lower autophagy gene expression than normal lung tissue in important autophagy genes such as ATG5, LC3A, LC3B, etc.
Figure 2. Decreased autophagy protein production in IPF patient lung tissue compared to controls. n=2. Important autophagy genes ATG5 and ATG12 are shown to have significantly reduced protein expression as compared to normal controls. β-Actin used as a loading control.

With autophagy mRNA and protein levels significantly reduced in IPF lung samples, we hypothesized that DNA methylation may be one cause.

Figure 3. DNMTs transcription levels are altered in IPF lung tissue samples. (n=3 per severity group). *p<0.005. Error bars consist of standard error. The more severe the type of IPF, the more DNMT is expressed. DNMT are DNA methyltransferases, and are the key enzymes involved in DNA methylation in the human body.
It has been established that in IPF lungs, autophagy has been repressed and promoter CpG island methylation levels are significantly higher than in normal lungs. Thus, inhibiting DNMT mRNA expression levels leading to a reduced promoter CpG island methylated autophagy gene signature may help restore autophagy function and induce normal collagen turnover in patients with IPF.
Figure 5. siRNA knockdown of DNMT-3B in normal lung fibroblasts (204) and severe IPF lung fibroblasts (191). Relative copy number (RCN) for DNMT-3B mRNA was calculated compared to housekeeping genes CAP1 and RPL4. Samples were then compared to a scrambled siRNA control (or no siRNA added) and the percentage of expression was determined.

![mRNA expression graph](image)

Figure 6. Autophagy gene expression with siRNA knockdown of DNMT-3B. 204=black, 191=red. Autophagy gene expression from severe IPF fibroblast cell line (red) as compared to normal fibroblasts (black). Knocking down DNMT-3B does not seem to effectively restore gene expression.
Due to these results, we thought it important to assay for baseline autophagy and DNMT mRNA levels in the cell lines as compared to the whole lung tissue samples (figure 8).

**Figure 7.** Knockdown of DNMT-3B has minimal effect on restoring autophagy function, *in vitro* (n=3). Green fluorescence-represents autophagosome vesicle formation. Blue stain represents cell nuclei of fibroblasts. More green indicates more autophagy function. Quantification of green pixels in all images indicates no significant difference when cells are treated with siRNA targeting DNMT-3B.
Discussion/Conclusion:

It is clear that the promoter CpG islands of essential autophagy-related genes are hypermethylated and transcriptionally repressed in IPF tissue samples compared to non-IPF control samples. This repression is sufficient to inhibit autophagy mRNA and protein expression levels and ultimately autophagy function, decreasing cellular senescence in IPF lung tissue (figures 1-4). However, in vitro studies of IPF and normal fibroblast cell lines suggest reduced DNMTs and autophagy-related gene expression levels (figure 8). Targeted knockdown of DNMT-3B had little effects on the regulation of autophagy mRNA expression levels in vitro,

Figure 8. Baseline mRNA expression for fibroblast cell lines. Differences in autophagy-related mRNAs expression between normal (204) and mild IPF fibroblasts (134) are larger than between normal and severe (191) (n=2). DNMTs mRNA expression is lower relative to IPF whole lung tissue levels.
as well as not having any impact on increasing autophagy function (figures 6,7). However, it is important to note the switch from human tissue (whole lung samples) to cell lines (fibroblast cell lines) in these experiments. One possible explanation for the unexpected differences between whole lung tissue samples and in vitro cell lines is the age of the cells. Aging is known to increase the incidence of IPF with over two thirds of patients with the disease over the age of 60\(^1\). Senescence, or the cellular state in which cells no longer respond to growth factor stimuli, is also known to increase with age\(^4\). Biomarkers for senescence include the tumor suppressor gene p16 and telomere length. It is possible that the cells used for the experiments simply were not old enough to replicate the results of the tissue, which come from older patients in severe stages of the disease. Preliminary results (data not shown) illustrate a relatively greater reduction in autophagy gene expression in older cells than their younger counterparts, along with a higher amount of DNA methyltransferase (DNMT) expression. However, this change is not nearly dramatic enough to account for the nearly 10-fold difference in expression levels between the tissue samples and the cell lines. Future studies will need to be performed to investigate why the cell lines seem to have different baseline levels than their tissue counterparts. For example, although we achieved 42 and 48% reduction in the expression of DNMT-3B mRNA in the normal and IPF fibroblast cell lines, autophagy gene expression was not completely restored to normal levels. We elected to utilize cell lines for knockout studies to demonstrate functional relevance and causation in a complete system. These experiments cannot be performed on frozen or fixed tissue in lung patient samples.

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